Chapter 3. MATERIALS AND METHODS

3.1. Isolation, biochemical identification, antibiotic susceptibility of bacterial isolates

3.1.1. Isolation of bacteria from clinical samples

IMS and Sum Hospital is a philanthropic and well-attended teaching hospital. Clinical samples (urine, stool, pus, blood, swabs and body fluids) were collected from both hospitalised patients and patients attending the OPD, aseptically (Figure 1). Routine microscopical examination of the samples was done to check the presence of bacteria and pus cells. The samples were also subjected to Gram-staining and simultaneously cultured on suitable media to check for the presence/growth of the bacteria (Figure 2 A, B). This study was approved by the institutional ethical committee of IMS and Sum Hospital.

Figure 1 A, Sterilised swab stick; B, Urine sample.

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3.1.2. Identification of bacteria

All the collected specimens except urine were inoculated onto MacConkey and blood agar plates. The urine samples were inoculated on cystine lysine electrolyte deficient (CLED) agar plates. Those inoculated plates were incubated aerobically at 37°C for 24 h. On the other hand, collected blood samples were incubated at 37°C overnight to check the growth of microorganisms. After incubation, those blood samples were inoculated on blood, chocolate and MacConkey agar plates and plates were kept in the same incubator. These blood samples were inoculated at second, fourth and seventh day of collection. Bacteria were identified basing on the colony characters.

Strains of 3 GPs, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus faecalis*; 5 GNs, *Acinetobacter baumannii*, *Citrobacter freundii*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were isolated from clinical samples of hospitalized patients. Growth of isolated bacteria on suitable media, and biochemical identifications of isolated strains along with corresponding reference strains from Microbial Type Culture Collections (MTCC), Chandigarh, viz., with MTCC numbers *S. aureus* 7443, *S. pyogenes* 1928 and *E. faecalis* 439, *A. baumannii* 1425, *C. freundii* 1658, *P. mirabilis* 739, *P. vulgaris* 1771 and *P. aeruginosa* 1688 were done. For the identification of bacterial strains, along with individual colony characters and biochemical tests were considered.

3.1.3. Biochemical identification of bacteria

For pure-culture samples of GP, catalase and coagulase tests were performed.

I. Catalase test: On a clean grease free slide, a drop of 3% H_2O_2 was mixed with a loopful of test bacterial culture. Occurrence of effervescence indicated the presence of catalase enzyme (Figure 2C).
n. Coagulase test: A lump of test organism was emulsified with a drop of normal saline water (0.89%). A drop of human blood serum was added to the suspension; clumping of cells was observed within 10s, which confirmed the presence of bound coagulase enzyme. When a sample of GP cocci responded positively to both catalase and coagulase tests, it was confirmed to be *S. aureus* (Figure 2D).

But catalase negative colonies were cultured on blood agar to check for their haemolytic patterns, and bacitracin test was conducted. Catalase negative GP colonies having beta-haemolysis (complete haemolysis of erythrocytes) on blood agar and simultaneously sensitive to the bacitracin are identified as Group A streptococci or *S. pyogenes*. Catalase negative, alpha-haemolytic (partial or green haemolysis of erythrocytes) colonies were subjected to bile-esculin test (Figure 2E). The bile-esculin medium contains esculin and peptone for nutrition and bile to inhibit growth of GP bacteria, other than Group D streptococci or enterococci. Ferric citrate was added as a colour-indicator. Organisms, which split esculin molecules and use the liberated glucose to supply energy, release esculin into the medium. The free esculin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar-slant from dark brown to black. An agar-slant that was more than half darkened within 48 h of incubation was bile-esculin positive, for the confirmation of *E. faecalis*; but the alternative non-darkening of the agar was taken as the negative result (Forbes et al., 2007).

For pure-cultures of GN bacilli, the following tests were done. III. Oxidase test: A bacterial colony was rubbed onto a filter paper, impregnated with tetramethyl-p-phenylenediamine dihydrochloride and the dye indophenols; the zone of the filter paper turns blue/purple in the positive result, while the negative result is with no change of colour (Figure 2F).
IV. Indole test: To an aliquot of 5mL 48 h old grown culture (test culture), an aliquot of 0.5mL of Kovac’s reagent (p-dimethylaminobenzaldehyde, isoamyl alcohol and HCl) was
added. A formation of a cherry-red or purple-red ring at the interface of the broth culture and the reagent indicated the indole production from tryptophan by the test culture (Figure 2G).

V. Methyl red test (MR test): To an aliquot of 5 mL sterile MRVP broth (peptone 7 g, glucose 5 g, potassium phosphate 5 g, pH 6.9, the test culture was inoculated and incubated for 48 h at 37° C. To this culture, 5 drops of methyl red solution were added as an indicator. If the total solution turned red, the test was taken as positive for the formation of organic acids as products.

VI. Voges-Proskauer test (VP test): To an aliquot of 5 mL sterile MRVP broth, a loopful of the test culture was inoculated and the mixture was incubated for 48 h at 37° C. To this culture tube, 10 drops of VP I reagent (5 % α-napthol, in absolute alcohol) and 2-3 drops of VP II reagent (40 % KOH solution) were added and the mixture was allowed to stand for 15-20 min for the reaction to complete. The positive result was the appearance of red colour of the mixture, i.e., production of a neutral product, acetoin from the fermentation of glucose by the organism, and alternately yellow colour production indicated the negative result.

VII. Oxidation Fermentation Test (O/F Test: Hugh-Leifson glucose broth (HLGB) with glucose, bromocresol purple, as main component and pH 7.4 was prepared. A small amount agar was used to get semisolid media to facilitate stab. After sterilization, Inoculation was done by stabbing of loop. Over one set of tubes paraffin was poured to give anaerobic condition and then incubated for 24 h and the other set incubated. Colour changed from purple to yellow in both tubes: fermentative. Colour changed only in tubes without paraffin oxidative. Colour change in any tube microorganism is inert to the media

VIII. Citrate test: The test culture was inoculated onto a slant of Simon citrate agar that was incubated for 48 h at 37° C. The change of colour of agar from green to blue indicated that organism used citrate as the sole source of carbon (Figure 2H).
IX. Urease test: The test organism was inoculated onto a slant of Christensen’s urea agar (peptone, glucose, sodium chloride, mono-potassium phosphate, urea, phenol red, distilled water, and at pH 6.8). The hydrolysis of urea yielding ammonia gas increases the pH that changes color of the medium from off-white to pink/orange, the positive result (Figure 2I).

X. Triple-sugar-iron test (TSI test): Two or 3 drops of test broth culture were inoculated on TSI-agar slant and subsequently, a stab was made up to the butt of the slant. The tube was incubated at 37°C for 48 h; black color appearance indicated H₂S production (Figure 2K).

XI. Nitrate test: An aliquot of 5 mL of nitrite broth (peptone 5 g, beef-extract 3 g, KNO₃ 1 g and distilled water 1,000 mL) was inoculated with 1 drop of 24 h old broth test culture and was incubated for 48 h at 37°C. From the development of red color within 30 sec of adding a few drops of the reagent A (α-napthol 5 g in 1,000 mL of 30 % acetic acid) and reagent B (sulphanilic acid 5 g in 1,000 mL acetic acid) the positive result was inferred. No color change suggested the negative result. MTCC strain of each GP or GN bacterium was used as the reference control in each biochemical test (Forbes et al., 2007; Figure 2L).

XII. Carbohydrate fermentation tests: Phenol red broth is a general-purpose differential test medium typically used to differentiate GN enteric bacteria. It contains peptone, phenol red (a pH indicator), a Durham tube, and one carbohydrate (or sugar). Five different kinds of phenol red broths were prepared each containing one kind of sugar, i.e., glucose, lactose, maltose, mannitol and sucrose. Phenol red is a pH indicator, which turns yellow below a pH 6.8 and fuchsia/pink color above pH 7.4. The test bacteria were inoculated to the each broth/tube and were incubated at 37°C for 48 h. If the bacteria was able to utilize the carbohydrate, an acid by-product is created, which turns the media yellow. If the bacterium was unable to utilize the carbohydrate but does use the peptone, the by-product is ammonia, which raises the pH of the medium and turns it fuchsia/pink color. When the bacterium was
able to use the carbohydrate, a gas by-product may be produced; an air bubble will be trapped inside a Durham tube. If the bacteria are unable to utilize the carbohydrate, gas will not be produced, and no air bubble will be formed (Forbes et al., 2007).

XIII. Motility test by hanging drop method: Motility test agar is a semisolid medium used for the detection of motility bacterial motility, which can be observed directly from examination of the tubes following incubation. Growing colony spreads out from the line of inoculation, if the bacteria are motile. Highly motile bacteria have growth throughout the tube. Growth of non motile organisms only occurs along the stab line. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile) (Figure 2M).

XIV. Phenylalanine deaminase test: To determine ability of bacteria to deaminate phenylalanine to phenyl pyruvic acid (PPA). Inoculate a medium containing phenylalanine is with a bacterial culture and incubate at 37° C for 18 to 24 h. Add a few drops of FeCl₂ solution. Green colour is produced in the formation of PPA. Green colour of the media was positive and colour remain unchanged was negative (Figure 2N).

3.1.4. Antibiotic susceptibility test by Kirby-Bauer's method
All bacterial strains including MTCC standard strains were subjected to antibiotic sensitivity tests by the Kirby-Bauer's/ disc-diffusion method, using a 4 mm thick Mueller–Hinton (MH) agar (HiMedia, Mumbai) medium, in duplicates. An aliquot of 0.1 mL of 0.5 McFarland equivalents, approximately from an exponentially growing culture was spread on agar for the development of lawn of a bacterium at 37° C in a BOD incubator (Remi CIM-12S). Further, on the lawn-agar of each plate, 8 high potency antibiotic discs (HiMedia) of 16 prescribed antibiotics were placed, separately at equal distances from one another. Plates were incubated for 18 h at 37°C and were examined for size of zones of inhibition around each disc, following the standard antibiotic susceptibility test chart of Clinical Laboratory Standard
Institute (CLSI) guidelines (CLSI, 2011). Experiments were done three times and data of the third set of experiments were presented (Forbes et al., 2007).

For GN bacteria; ampicillin (AMP) (10 µg/disc), amoxicillin-clavulanic acid (AMC) (20/10 µg/disc), gentamicin (GEN) (30 µg/disc), amikacin (AK) (30 µg/disc), norfloxacin (NX) (10 µg/disc), levofloxacin (LV) (10 µg/disc), ciprofloxacin (CIP) (5 µg/disc), nitrofurantoin (NIT) (30 µg/disc), ceftriaxone (CTR) (30 µg/disc), ceftazidime (CAZ) (30 µg/disc), piperacillin/azobactam (PIT) (100/10 µg/disc), cefoperazone (CS) (75 µg/disc), cefoperazone sulbactam (CFS) (75/10 µg/disc), cefepime (CPM) (30 µg/disc) were used.

For GP bacteria; ampicillin (AMP) (10 µg/disc), amoxicillin-clavulanic acid (AMC) (30 µg/disc), gentamicin (GEN) (10 µg/disc), amikacin (AK) (30 µg/disc), ciprofloxacin (CIP) (5 µg/disc), cefotaxime (CTX) (30 µg/disc), linezolid (LZ) (30 µg/disc), azithromycin (AZ) (30 µg/disc), oxacillin (Ox) (1 µg/disc), vancomycin (VA) (30 µg/disc), piperacillin (PI) (10 µg/disc), penicillin-G (P) (10 µg/disc) were used. The plates were then incubated at 37° C for 24-48 h. Diameters of the zone of inhibition around the disc were measured using a zone measurement scale (caliper) and the isolates were classified as sensitive, intermediate, and resistant according to the standardized table supplied by the CLSI guidelines.

3.2. Prevalence of methicillin resistant S. aureus

3.2.1. Antibiotic sensitivity of S. aureus isolates

Clinical samples were collected for isolation of S. aureus strains from patients attending OPD, as well as IPD patients of Sum Hospital, Bhubaneswar. Over a period of 30 months (November 2009- April 2012), only 7234 samples were found to have one or other bacterial pathogen. GP in clusters were cultured on nutrient agar and blood agar media; when cultured on nutrient agar, butyrous, glistening, round, elevated, medium-sized colonies with golden colour (due to the presence of triterpenoids or carotenoids in cell membrane), and on blood agar with yellow coloured, round and elevated colonies with β-haemolysis were seen; those
were taken to be *S. aureus*. The strain of Microbial Type Culture Collection (MTCC) *S. aureus* 7443 was used as the reference control. For confirmation, the colonies were streaked on gelatin mannitol salt agar medium and were incubated at 37° C, for 48 h for the growth of yellow colonies (Forbes et al., 2007).

### 3.2.2. Detection of MRSA by chromogenic agar media test

Pure clinical isolates of *S. aureus* were streaked onto methicillin resistant *S. aureus* agar media (Hichrome-MeReSa agar media, Himedia) and were incubated for 24 h at 37° C. Colonies appearing blue after the incubation period were detected as MRSA strains, and non-MRSA strains appeared white, according to guidelines of the media supplier.

### 3.2.3. Cefoxitin disc diffusion test

All the isolates were subjected to cefoxitin 30 μg/disc. A 0.5 McFarland standard suspension of an isolate was made and lawn culture was done on Muller-Hinton agar (MHA) plate. Plates were incubated at 37° C for 18 h and inhibition-zone diameters were measured. A value of inhibition-zone diameter less than 22 mm was reported as oxacillin resistant and that more than 21 mm was considered as oxacillin sensitive (Swenson et al., 2001).

### 3.2.4. Inducible clindamycin resistant *S. aureus*

Isolates that were 'Er-r, Cd-s' were tested for inducible Cd-r, by susceptibility to clindamycin 2 μg/disc and erythromycin 15 μg/disc levels along with the reference strain, according to CLSI criteria (CLSI, 2011). Erythromycin and clindamycin discs (HiMedia) were placed 17±2 mm apart (edge to edge) on a MHA plate, incubated at 37° C for 18 h and D-test positivity was identified by the flattening of clindamycin zone between erythromycin and clindamycin discs to assume the D-shape. Any isolate with 'Er-r, Cd-r' was considered as constitutive MLSB resistant strain (Jorgensen et al., 2004). *S. aureus* MTCC was used as methicillin-susceptible control strains.
3.2.5. Determination of MIC values
A 96-well micro-titer plate was used to determine the MIC values of oxacillin in broth cultures, for 25 selected strains, of each which MRSA, MSSA. An exponential culture of a test strain of S. aureus in Muller-Hinton (MH) broth (HiMedia) were suitably diluted with the normal saline solution to obtain the level of equivalent to the 0.5 McFarland standard. TTC (2, 3, 5-triphenyltetrazolium chloride) was used as an indicator of bacterial growth. To an aliquot of 20 μL overnight grown test culture, an aliquot of 100 μL of the antibiotic stock solution of concentration 512 μg/mL and an aliquot of 100 μL of MH and BHI broth were added to the second well of the micro-titer plate. This solution was serially diluted at each successive well till the final concentration of 0.25 μg/mL antibiotic in the 12th, the last well was obtained. In the last step, an aliquot of 5 μL 0.5 % TTC was added to each well and the micro-titer plate was incubated at 37° C for 18 h. Wells were examined for the development of the pink colour that in a well indicated the bacterial growth, and the absence of the pink colouration was taken as the growth inhibition and that was the MIC value (Eloff, 1998). Obviously, the first well of the micro-titre plate was the control without any antibiotic.

3.2.6. Hi comb MIC test for oxacillin
MICs of the antimicrobials for the isolates were cross checked by the Hi comb MIC test (Hi Media) performed according to the protocols supplied by the manufacturer. The S. aureus MTCC 7443 strain was the reference strain. The MIC was the value at which, the zone converges on the comb-like projections of the strips. It was a rapid and reliable method for the determining the antimicrobial susceptibility of different microorganisms against particular antibiotic. The zone of inhibition was in the form of an ellipse. MIC value would be the value at which, the zone convenes the comb like projection of the strip and not at the handle, if there was no zone of inhibition observed, then the strain was considered as resistant to that antibiotic.
3.2.7. Statistical analysis
The statistical analysis was performed using the Statistical Package for Medical Science SPSS version 20 (IBM Corp., 2011) and Microsoft Excel.

3.3. Prevalence of vancomycin resistant *E. faecalis*
Clinical samples were collected for isolation of *E. faecalis* strains from patients attending OPD, as well as patients admitted into wards, cabins, ICU and neonatal intensive care unit (NICU) of Sum Hospital, Bhubaneswar. Over a period of 30 months (November 2011- April 2013), only 7634 samples were found to have one or other bacterial pathogen. GP cocci in clusters were cultured on nutrient agar and blood agar media; for pure-culture samples of these GP, catalase and coagulase tests were performed, as detailed previously.

Catalase negative colonies were cultured on blood agar to check for their haemolytic patterns, and bacitracin test was conducted. Catalase negative, alpha-haemolytic (partial or green haemolysis of erythrocytes) colonies were subjected to bile-esculin test. The bile-esculin medium contains esculin and peptone for nutrition and bile to inhibit growth of GP bacteria, other than group D streptococci or enterococci. Ferric citrate was added as a colour-indicator. Bacteria split esculin molecules and use the liberated glucose to supply energy, release esculin into the medium. The free esculin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar-slant from dark brown to black. An agar-slant that was more than half darkened within 48 h of incubation was bile-esculin positive, for the confirmation of *E. faecalis*; but the alternative non-darkening of the agar was taken as the negative result (Forbes et al., 2007). The MTCC *E. faecalis* 439 strain was used as the reference control.

3.3.1. Detection of VRE by vancomycin screen agar plate method
Screening for vancomycin resistance was done by the screen agar method on both MH agar and brain heart infusion (BHI) agar (HiMedia). Vancomycin screen agar plate was prepared
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by an addition of 6 mg/L vancomycin to BHI agar and MH agar. Inoculum suspension was prepared by transferring colonies of *E. faecalis* from an overnight-growth culture on nutrient agar plate to sterile saline to produce a suspension that matched the turbidity of the 0.5 McFarland standards. An aliquot of 0.1 mL of the suspension was spread on to vancomycin screen agar plates and were incubated for 24 h at 37° C. Any visible bacterial growth indicated the vancomycin resistance (Tenover et al., 2001). *E. faecalis* MTCC 439 were used as methicillin- and vancomycin-susceptible control strains, respectively.

3.3.2. Inducible clindamycin resistant *E. faecalis*
Isolates that were ‘Er-r,Cd-s’ were tested for possible inducible Cd-r, to probe susceptibility to clindamycin 2 µg/disc and erythromycin 15 µg/disc levels, along with the reference strain, according to CLSI criteria as described earlier in 3.2.4.

3.3.3. Determination of MIC values
A 96-well micro-titer plate was used to the MIC values of vancomycin in broth cultures, for 25 selected strains, of each which VRE and VSE was determine as described earlier in 3.2.5.

3.3.4. Hi comb MIC test for vancomycin
MICs of the antimicrobials for the isolates were cross checked by the Hi comb MIC test (Hi Media) performed according to the protocols supplied by the manufacturer as done previously. The *E. faecalis* MTCC 439 was the reference strain.

3.4. Prevalence of ESBL producing Gram negative bacteria
Detection of ESBL strains were conducted over a period of 15 months (February 2012 to April 2013) in Department of Microbiology of the institute. Clinical samples were collected for isolation of strains from patients attending OPD, as well as patients admitted into wards, cabins, ICU and NICU of the hospital.

3.4.1. Detection of ESBL producers by double disc synergy test
The (DDST) was used to detect the ESBL producing activity of selected bacterial strains on a lawn culture on a MHA plate. The augmentin 30 µg disc (20 µg amoxicillin and 10 µg
clavulanic acid) in the middle was flanked by a disc of ceftazidime 30 μg and a disc of cefotaxime 30 μg (both third generation cephalosporins, 3GC), at 30 mm apart on a lawn culture. The set up was done in triplicate and was incubated at 37° C for overnight for observation of inhibition zones. Indeed, the augmentin disc inhibits the action of the ESBL enzyme; eventually, an inhibition zone is formed from a peripheral disc towards the middle, due to the synergistic action of the augmentin and the corresponding cephalosporin disc. In general, if the bacteria are resistant to both cephalosporins, because of the production of the ESBL enzyme, the action of augmentin deactivates the enzyme with a consequent reactivation of a cephalosporin resulting in the extension of the inhibition zone (Vercauteren et al., 1997).

Simultaneous tests with non-ESBL-producing MTCC strains of each isolated bacterium were used in parallel: C. freundii (MTCC 1658), P. mirabilis (739), P. vulgatis (MTCC 1771), P. aeruginosa (MTCC 10866) and A. baumannii (MTCC 1425).

3.4.2. Determination of MIC values
ESBL positive MDR strains were further used for ascertaining MIC values due to 3GC antibiotics (cefotaxime, ceftazidime, ceftriaxone, cefpodoxime and cefexime). A standard MTCC bacterial strain was taken as the negative control for each bacterium. The MIC value of each antibiotic against ESBL producers and non-producers were determined in a 96-well microtitre plate as described earlier in 3.2.5.

3.5. Monitoring antibacterial activities of plants
3.5.1. Collection and Identification of the plant material
Plants reported (listed in Table 1) were collected from Kalahandi forest pockets of Odisha during July 2009 to December 2010. The plant material were identified using the suitable flora book (Haines, 1924).
3.5.2. Preparation of cold plant extracts

Collected mature leaves/barks of plants were crushed to powders, after shade drying. A lot of 5 g of powder of a sample was dissolved in an aliquot of 25 mL of double distilled water and was sterilized for 30 min, before incubation at 4°C for 72 h, with intermittent stirring. These steps were repeated for each plant sample. Water extracts were used directly for monitoring antibacterial properties *in vitro*. For an ethanol extract, a lot of 5 g of each powdered plant material was soaked in an aliquot of 25 mL 80% ethanol for 72 h with the usual hand-shakings, and the mixture was filtered. The alcohol-filtrate was concentrated in a rotary evaporator at 40°C, till a sticky mass was obtained that was weighed and dissolved in an aliquot of 1 mL 10% v/v DMSO. For each plant sample, these steps were repeated and both extracts were stored at 4°C until further use (Figure 3).

![Figure 3 Preparation of plant extracts by cold percolation method.](image)

3.5.3. Preparation of hot plant extracts

For the hot extraction, a 40 g lot of powdered plant material (both leaves and bark, separately) were dissolved in a volume of 400 mL (approximately) of an organic solvent in a Soxhlet apparatus. According to the ‘successive extraction procedure’, eight non-polar to polar solvents, petroleum ether, ethyl acetate, chloroform, n-hexane, acetone, methanol, ethanol and water were used; extractions were carried out at 40-60°C, depending upon the
boiling point of the organic solvent in use. About after 40 cycles or siphons with petroleum ether, the liquid extract was collected at the bottom flask and was dried using the rotary evaporator until a semisolid mass was obtained, which was stored in 10 % dimethyl sulfoxide (DMSO) solution, at 4° C for use. The leftover plant material in the Soxhlet apparatus was further oven dried at 40° C and was reused for another extract using the next solvent in the succession list, cited above (Figure 4).

![Figure 4 Hot extraction using Soxhlet apparatus.](image)

3.5.4. Agar-well diffusion method for antibacterial assays of plant extracts

Antibacterial activities of different solvent-extracts of all the plants were done by the agar-well diffusion method. One strain from each bacterial species having resistance to maximum numbers of antibiotics was used, for monitoring antibacterial activities of plant extracts. Bacterial lawns were prepared with agar being 6 mm thick that was fully punched and 6-8 wells were prepared, when a lawn was 30 min old, and each well was based by 50 µL molten MH agar, in duplicates. Further, wells were filled with 100 µL aliquots of 30 mg/mL solvent-
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extracts of the plant, diluted from the original stock of plant extracts of individual organic solvents with the aqueous extract, by 10 % DMSO solution. Plates were incubated at 37° C for 18-24 h. Antibacterial activities were evaluated by measuring the diameter values of zones of inhibition. Experiment of each solvent-extract was conducted thrice and data of the third repeated experiment are presented. An aliquot of 100 µL of chloramphenicol 30 µg/mL with an average diameter of zone of inhibition of 21 mm and 10 % DMSO solution were reference controls; 10 % DMSO solution had no antibacterial activity (Perez et al., 1990).

3.5.5. Determinations of MIC and MBC values
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of three active hot and cold plant-extracts, prepared with organic solvents were determined, by suitable dilutions from original stock solutions of each extract, leaves and barks, for concentrations, 0, 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg plant-extract/mL in aliquots of 10 % DMSO solution. Separate experiments were conducted for each solvent-extract. An aliquot of 80 µL of each dilution of a solvent-extract was released to a well on a 96-welled (12x8) micro-titer plate, along with an aliquot of 100 µL MH broth (HiMedia), an aliquot of 20 µL bacterial inoculum (10⁹ CFU/mL) and a 5 µL-aliquot of 0.5 % 2,3,5-triphenyl tetrazolium chloride (TTC). After pouring all the above materials to a well, the micro-titre plate was incubated at 37° C for 18 h. The development of pink colouration due to TTC in a well indicated bacterial growth and the absence of the colouration was taken as the growth inhibition. The first well of the micro-titre plate was the control, without any plant extract (Eloff et al., 1998) The MIC value was noted at the well, where pink colour was not manifested. Further, bacteria from each well of the micro-titre plate were sub-cultured on nutrient agar; the level of dilution, where no bacterial growth on the nutrient agar was observed, was noted as the MBC value. Results of the second repeated experiment were presented.
3.6. Preliminary phytochemical analyses

I. Test for reducing sugars: Aliquot of 2 ml of the aqueous extract in a test tube was mixed with 5 ml mixture of equal volumes of Fehling's solutions I and II and the mixture was heated in a water bath for about 2 minutes. A brick-red precipitate between aqueous extract and Fehling solution I and II indicated the presence of reducing sugars.

II. Test for saponins: A lot of 0.5 g of an extract was dissolved in an aliquot of 10 mL of distilled water in a test-tube, which was shaken vigorously for 30 seconds and subsequently was allowed to stand for 45 min. The appearance of frothing on warming the lot indicated the presence of saponins.

III. Test for flavonoids: To a portion of the dissolved extract, a few drops of 10 % ferric chloride solution were added. Green or blue colouration indicated the presence of flavonoids.

IV. Test for steroids/terpenes: A lot of 500 mg of the concentrated mass of the plant-extract from the rotary evaporator was dissolved in an aliquot of 2 mL of acetic anhydride and the mixture was cooled at 0 to 4°C, to which a few drops of 12 N sulphuric acid were carefully added. Colour change from violet to blue-green indicated the presence of a steroidal nucleus.

V. Test for tannins: A lot of 0.5 g of the extract was dissolved in 5 mL aliquot of water followed by addition of a few drops of 10 % FeCl₃ solution. A blue-black, green, or blue-green precipitate indicated the presence of tannins.

VI. Test for alkaloids: A lot of 0.5 g of plant-extract was stirred with an aliquot of 5 mL 1 % HCl on a steam bath, and the mixture was filtrated; to an aliquot of 1 mL of the filtrate, a few drops of Mayer's reagent (1.36 g HgCl₂, 5 g KI in 100 mL distilled water) was added, and to another aliquot of 1 mL of the filtrate, a few drops of Dragendorff's reagent (two solutions in 1:1 ratio — 'solution A' with 0.85 g bismuth nitrate, 10 mL glacial acetic acid and 40 mL distilled water, and 'solution B' with 8 g KI in 30 mL distilled water) were added. Turbidity or precipitation in tubes due to either of these reagents indicated the presence of alkaloids.
VII. **Test for glycosides:** An aliquot of 5 mL of each extract was mixed with an aliquot of 2 mL of glacial acetic acid (1.048 g/mL), one drop of 1 % FeCl₃ solution and mixed thoroughly to which, an aliquot of 1 mL of 12 N H₂SO₄ was added. A brown ring at the interface indicated the presence of glycosides. All these tests were repeated for confirmation (Harborne, 1998; Sofowara, 1993). This procedure was followed for cold extracts of 70 plants.

3.7. **Work on most potent plants from preliminary screening**

3.7.1 **Analysis of physicochemical properties of leaves Woodfordia fruticosa (L.) Kurz**

The following physicochemical parameters of the plant *W. fruticosa* were assessed.

I. **Loss on drying:** A lot of 2 g of leaf powder of *W. fruticosa* in an evaporating dish dried in an oven at 105°C till constant weight was obtained. The weight after the total drying was noted; the percentage of loss on drying was calculated.

II. **Total ash:** A lot of 2 g powder of *W. fruticosa* (leaf) was taken in a silica crucible and ignited it by gradually increasing the heat to 500°C until it was white, indicating the absence of carbon. Ash was cooled in desiccators and weighed without delay.

III. **Acid insoluble ash:** To the crucible containing total ash, 25 mL of hydrochloric acid (70 g/L) was added; it was covered with a watch-glass and boiled gently for 5 min. The watch-glass was rinsed with 5 mL of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ash less filter paper and it was washed with hot water until the filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible; it was dried on a hot plate and ignited to constant weight. The residue was allowed to cool in desiccators for 30 min and then weighed without delay. Acid insoluble ash was expressed in mg per g of air dried material.

IV. **Water soluble ash:** To the crucible containing the total ash, 25 mL of water was added and boiled for 5 min. The insoluble matter was collected on an ash less filter paper. It was
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washed with hot water and ignited in a crucible for 15 min. Weight of insoluble matter was subtracted from the weight of total ash. The content of water soluble ash was calculated in mg per g of air dried material.

V. Determination of alcohol soluble extractive: Four grams of crude powder of *W. fruticos* was macerated with 100 mL of alcohol in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105° C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

VI. Determination of water soluble extractive: Four grams of crude powder of the plant was macerated with 100 mL of water in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105° C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

VII. Determination of methanol soluble extractive: Four grams of crude powder of *W. fruticos* was macerated with 100 mL of methanol in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105° C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

VIII. Determination of petroleum ether soluble extractive: Four grams of crude powder of *W. fruticos* was macerated with 100 mL of petroleum ether in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105° C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.
IX. Solubility: The quantitative solubility test of methanol extract of the plant was determined in different solvents. 50 mg of extract was weighed for solubility test for different solvents. The extract was added in each solvent until saturated solution developed. Solubility was calculated in mg/mL.

X. Determination of pH: The methanol extract of *W. fruticosa* was dissolved in distilled water and was kept in a water bath for 20 min. It was then filtered and the pH of the filtrate was noted with the help of a Systronic pH meter (pH YorkoYSI601).

XI. Determination of microorganisms: Total bacterial and total fungal counts as well as specific count for *E. coli, S. typhi* and *P. aeruginosa* for crude powder and methanol leaf extract of *W. fruticosa* was carried out using reported methods.

3.8. Bioassay guided fractionation, antibacterial activity and GC-MS analysis of *W. fruticosa* leaves

![Figure 5 Liquid–liquid fractionation of *W. fruticosa*.](image)

3.8.1. Bioassay guided fractionation of methanol leaf-extracts of *W. fruticosa*

In hot extraction method, dried leaf powders of *W. fruticosa* were used for obtaining the methanolic extract with soxhlet apparatus for 24 h; the extract filtered and the filtrate was
dried in vacuum. The methanol crude extract was subjected to bioassay-guided fractionation by solubilising in water and sequential partition with n-hexane, chloroform, ethyl acetate, dichloromethane and n-butanol, while the end product was termed as methanol-fraction (Figure 5). Each collected fraction was concentrated under reduced pressure for a dark residue (Schrader et al., 2013).

3.8.2. Antibacterial activity test of 6 different solvent fractions of *W. fruticosa* by agar-well diffusion method

By agar-well diffusion method, antibacterial activities of the 6 different solvent fractions of *W. fruticosa* were done, as described previously. Linezolid 30 μg/mL and imipenem 10 mg/mL were used as reference controls for all the GP and GN MDR bacteria in determination of antibiograms, respectively.

3.8.3. Determinations of MIC and MBC values of n-butanol fraction of *W. fruticosa*

MIC and MBC values of the active n-butanol fraction of *W. fruticosa* were determined, as detailed previously in 3.5.5.

3.8.4. GC-MS analysis of n-butanol fraction of *W. fruticosa*

The GC-MS analysis of the n-butanol fraction was carried out using a GC-MS unit equipped with a VF-5 ms fused silica capillary column of 30 m length, 0.25 mm diameter and 0.25 μm film thickness. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.99 %) was used as a carrier gas at the constant flow rate of 1.51 mL/min. Temperatures of injector and mass transfer line were set at 200 and 240° C, respectively. The oven temperature was programmed from 70 to 220° C at 10° C/min, held isothermal for 1 min and finally was raised to 300° C at 10° C/min. Aliquots of 2 μl of respective diluted samples were manually injected in the split less mode, with split ratio of 1:40 and with mass scan of 50-600 amu. The total running time of GC-MS was 60 min (Hossain et al., 2013).
3.8.5. Identification of compounds

The phytochemical components of the biologically active fraction, the ethyl acetate fraction, were identified by comparison of their mass spectra fragmentations and retention indices with those stored in databases, NIST08.LIB (Stein SE National Institute of Standards and Technology, Mass Spectral Database and Software, Version 3.02, Gaithersburg, MD, USA, 1990) and WILEY8.LIB (McLafferty, 1989), and also with the published literatures.

3.9. Host toxicity testing of methanolic extracts of *W. fruticosa*

3.9.1 Collection of lymphocytes

Umbilical cord blood (UCB) was collected in a sterile 15 or 50 mL size falcon tube (Tarson, Kolkata), with an aliquot of 100 or 250 μL 1,000 IU heparin (HiMedia), immediately after the delivery of an infant and the blood sample (less than 15 to 50 mL) was stored at 4°C till use. Lymphocytes were isolated immediately or within the best 24 h after the collection.

For the isolation of lymphocytes, the collected UCB sample was diluted with an equal volume of phosphate buffered saline (PBS) solution. Further, the mixture was loaded carefully into a centrifuge tube for over-layering with lymphocyte separating medium (LSM, HiMedia), which was one-third the total volume of the mixture. The mixture was centrifuged at 1800 rpm for 25 min at 22-24°C, as a result four heavy to light layers, red blood cells (RBC), LSM, buffy coat and plasma were seen. The buffy coat layer with mononuclear cells was taken out carefully from the tube. To the separated cells of the buffy coat layer, after the addition of another aliquot of PBS for the 1:1 ratio, re-centrifugation at 2000 rpm for 5 min was done. The pellet of lymphocytes was taken for culturing and cell counts were done using a haemocytometer (Broxmeyer et al., 1989).

3.9.2. Growth of lymphocytes

After separation, UCB-derived lymphocytes were diluted to the density of 1x10⁶ cells/mL with a required volume of Dulbecco’s modified Eagle’s medium (DMEM-low-glucose,
HiMedia), and were loaded into a 6-well culture plate (Tarson), which contained 15% fetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin and 1% sodium pyruvate, along with graded concentrations of plant extract for growth. The stock solution was prepared by dissolving 100 mg of plant extract in an aliquot of 100 mL of triple distilled water for the concentration of 1000 μg/mL and the stock solution was stored at 4°C, for further use. The volume of 2 mL in total was maintained in each well of the culture plate with plant extract solution. The cells were incubated with different concentrations of methanolic extract of *W. fruticosa* (0, 25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 μg/mL) in an incubator at 37°C in 5% atmospheric CO₂ concentration for 24 h for growth.

3.9.3. Monitoring toxicity by MTT assay
The viability of lymphocytes grown in the presence of plant extract was by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. The MTT solution was prepared at the concentration of 5 mg/mL in PBS. After 24 h of a toxin treatment in 6-well culture plate, 80 μL of MTT solution was added to each well to study the toxicity effect. The plate was kept in an incubator (37°C, 5% CO₂) for 4 h. Then, it was found that the media containing the cells and the toxin converted to blue colour after incubated with MTT. Then the lot was gently centrifuged at 1000 rpm for 10 min at 22°C. The supernatant was removed and the pellet was dissolved by 1 mL 100% DMSO and kept in the incubator for 1 h. A purple colour was seen. Optical density was measured with a spectrometer at the 570 nm wavelength (Raheel et al., 2013).

Percentage of cell density = 100 X (OD_sample – OD_blank)/OD_control, MTT in DMSO solution was taken as the blank. Probits of observed lethality percentage values were used for analysis of toxicity.
3.9.4. Comet assay

Lymphocytes were cultured with different concentrations of *W. fruticosa* methanol extract and the harvested cells were used by the neutral comet assay technique to study DNA damages. Slides were coated with 1 % agarose and allowed for air drying. Pellets of lymphocytes, obtained by centrifugation of cultured cells were washed with PBS and the pellet was mixed with three times the cell volume of the pellet with low melting point agarose (LMPA) 1 % in sol state. The mixture of cells and LMPA sol was placed over the agarose coated slide that was kept at 4°C for 10 min until the slide got dry. The dried slides were submerged into a pre-cooled lysing solution of the mixture of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 7.4, 1 % Triton-x 100 and 10 mM dithiothreitol (DTT) and the mixture was kept at 4°C in dark for about 2 h. The slides were subsequently removed and placed in an electrophoresis buffer with 500 mM NaCl, 100 mM Tris, 1 mM EDTA, 0.2 % DMSO, for 20 min. The slides were transferred to a horizontal gel electrophoresis chamber with fresh electrophoretic buffer. Electrophoresis was carried out at 10v and 250 mA for 60 min. After the electrophoresis, the slides were washed in PBS for 5 min and were placed in a neutralizing solution with 50 % ethanol and 20 mM Tris for 5 min, and again the slides were washed in PBS. After 5 min the slides were stained with ethidium bromide solution. The slides were observed under the fluorescence microscope at 400X and the comets were scored. Probits of observed lethality percentage values calculated from percent values of observed comets due to plant extract toxicity (Cortes-Gutierrez et al., 2011).

3.10. *In vitro* combination-efficacy of vancomycin and leaf extract of *W. fruticosa* against multidrug resistant *S. aureus*

3.10.1 Preparations of plant extracts

Leaves of *W. fruticosa* were dried at 37°C for 24 h in an incubator and were further shade-dried for 15 days. Dried leaves were powdered and the powder-mass was stored

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in air-tight polythene packs till use. In a Soxhlet apparatus, methanolic extraction of leaves was carried out with a lot of 40 g of powder-mass in the extractor and a volume of 400 mL methanol was used during 24 h of soxhletion, in about 40 siphons of extraction. After filtration, the extract was concentrated in a rotary evaporator. The obtained sticky-mass was dried in a desiccator for a solid mass, black in colour, which was stored in a suitable volume of 10 % DMSO at concentration of 50 mg/mL, at 4°C.

3.10.2. Antibiotic sensitivity pattern of isolated *S. aureus*
The disc diffusion method was used for the antibiotic sensitivity test, using a 4 mm thick MHA medium, as detailed previously.

3.10.3. Determination of MIC and MBC values of the leaf-extract and vancomycin
A powder (injective form) of the antibiotic, vancomycin was dissolved in an appropriate volume of water for a stock solution of 1 mg/mL; the plant extract was maintained at 50 mg/mL. The sensitivities of *S. aureus* to the methanolic leaf-extract and vancomycin were evaluated by determining MIC values. Serial dilutions of the methanolic-extract were released in succession to wells on a row in a 96-welled (12 x 8) microtitre plate; an aliquot of 80 μL of the leaf-extract was given to the first well with an aliquot of 100 μL of nutrient broth (HiMedia); from the first well again, an aliquot of 80 μL was drawn and transfer to second well with 100 μL of nutrient broth; the rest of the wells received 80 μL content of the previous well, in succession for the total of 11 wells; an aliquot of 20 μL bacterial inoculum (10^5 CFU/mL) and a 5μL-aliquot of 0.5 % of TTC were added to each well. The 12th well did not receive the plant-extract and served as the control. After pouring all the above to each well, the micro-titer plate was incubated at 37°C for 18 h. A pink colouration due to TTC in a well indicated bacterial growth and the absence of pink colour was taken as growth inhibition. The MIC value was noted at the well with lowest level, where the pink colour was not manifested. Further, bacterial content from each well of the micro-titre plate was sub-
cultured on nutrient agar plates; from ascertained MIC levels and above, the level of dilution, where no bacterial growth on the nutrient agar plate was observed, was noted as the MBC value. This method was repeated to determine MIC and MBC values of the antibiotic.

3.10.4. Checkerboard technique for combination activity of leaf-extract and vancomycin
To evaluate the synergistic/antagonistic interaction action of the leaf-extract with vancomycin, the checkerboard procedure was followed. In this method, the leaf-extract and the antibiotic were given in the wells of the microtitre plate in serially proportionate combinations along with nutrient broth, bacterial (a MDR strain) inocula and TTC, for a visible growth of the bacterium. The combinations of ‘leaf-extract and vancomycin’ were prepared in the following proportion, 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10. Each proportion-lot was adjusted to 1.0 mL in an eppendorf tube; the solution of each tube was dispensed to a row of 11 wells in the microtitre plate, serially. As described herein, each well finally had an aliquot of 100 μL of nutrient broth, 20 μL aliquot of bacterial inoculum and a 5 μL-aliquot of TTC, for the growth of the bacterium. The combinations were checked for MIC and MBC values with the antibiotic, as described above. Interaction was assessed algebraically by determining FIC indices; FIC index = FIC of extract + FIC of ceftriaxone. Additionally, fractional bactericidal concentration (FBC) index values for both the leaf-extract and the antibiotic were computed. The FIC index value was interpreted as: (i) a synergistic effect, when it was lesser than 0.5; (ii) an additive or indifferent effect, when it was more than 0.5 and lesser than 1; and (iii) an antagonistic effect, when it was more than 1.0.

3.10.5. Inhibition zone of different concentration of leaf-extract of W. fruticosa
Inhibition zone (IZ) of the methanolic leaf-extract of W. fruticosa against S. aureus was determined by the agar-well diffusion method as described previously.